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THE CHEMISTRY OF ALLERGENS

XIV. Effect of Heat and pH on the Precipitin Reaction and Reagin Neutralizing Capacity of the Castor Bean Allergen, CB-1C

MAXIMUM industrial utilization of castor bean pomace has been prevented because the allergen contained in castor beans has unusually potent sensitizing capacity for those exposed to the dust, and because this allergen provokes severe asthma in hypersensitive individuals. The problem of industrial inactivation of the allergen has been complicated by the fact that the allergen is stable to heat treatment which destroys the potent toxalbumin, ricin.

The first recorded case of hypersensitivity to castor beans was described in 1914 by Alilaire who attributed the allergenic activity to ricin.[1] The first recorded case of occupational castor bean sensitivity was that of a chemist working at the United States Department of Agriculture described by Bernton.[2] Since then, many cases of hypersensitivity to castor beans have been reported. Figley and Elrod[3] described the first endemic asthma involving thirty cases within a one-mile radius of a castor bean processing plant. Ordman[4] described an outbreak of asthma in South Africa affecting

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Previous paper in this series, "The Chemistry of Allergens XIII. Ion-Exchange Fractionation of the Cottonseed Allergen and Immunological Properties of the Products" by Joseph R. Spies, Dorris C. Chambers and E. J. Coulson. *Archives of Biochemistry and Biophysics,* 84:286 (Oct.) 1959.

over two hundred persons caused by dust from a castor oil processing plant. Castor bean allergy due to contamination of burlap bags and of green coffee also has been reported.[5-7] The literature on this subject is reviewed by Ordman and need not be repeated here.

The isolation and properties of the principal allergen of castor beans, CB-1A or CB-1C (see material used) have been described in previous papers from this laboratory.[8-13] CB-1C is a polysaccharide protein belonging to the natural proteose classification which represented 1.8 per cent of ether defatted domestic castor beans and 0.33 per cent of commercial pomace by isolation. The allergenic and antigenic specificities of CB-1C are attributed to the protein component. CB-1C is water soluble, stable in boiling water, precipitated by 75 per cent ethanol and soluble in basic lead acetate solution. CB-1C is composed of known amino acids with relatively high arginine and glutamic acid contents and no tryptophan. CB-1C contains no ricin and is nontoxic.

The object of this paper is to describe the effect of heating CB-1C in buffered solutions at pH values of 4, 5, 6, 7, 8, 9, and 10 at temperatures of 100, 115, and 130° C for various periods of time: (1) on the precipitating capacity with castor bean allergen rabbit antiserum, and (2) on the *in vivo* reagin neutralizing capacity by a passive transfer method with serum from a castor bean sensitive person.

MATERIAL USED

Castor Bean Allergen, CB-1C.—CB-1C was isolated from commercial Brazilian castor bean pomace by the "1C" procedure.[13] The nitrogen content was 13.6 per cent, air-dried basis. The difference between CB-1A and CB-1C is in the method of removal of lead in the isolation procedure. Lead was removed with sodium carbonate in the preparation of CB-1A and with hydrogen sulfide in the preparation of CB-1C. The two preparations were chemically and immunologically indistinguishable.

Castor Bean Allergen, CB-13E.—Some unimportant polysaccharide and some denatured protein were eliminated from CB-1A by precipitation with picric acid and recovery of active fraction CB-13 by removal of the picric acid from the precipitate. CB-13E was prepared by dialysis of CB-13 by essentially the same procedure used to obtain the corresponding fraction CS-13E from the cottonseed allergen, CS-1A.[14] Eleven grams of CB-13 were obtained from 21.1 g of CB-1A. Three and four tenths grams of CB-13E, and 4.9 g of combined dialysates were obtained by dialysis of 10.8 g of CB-13. CB-13E contained 17.95 per cent nitrogen and 2.48 per cent polysaccharidic carbohydrate (ash-water-free basis).

Rabbit Antiserum.—Rabbits were immunized to CB-13E by a series of inoculations of antigen in Freund's complete adjuvant[15-17] followed by a series of intraperitoneal injections of antigen in saline according to the following schedule: Twelve rabbits were given four weekly subcutaneous injections of 4 mg of CB-13E contained in 0.5 ml of Freund's adjuvant.

Serum prepared from blood drawn two weeks after the fourth inoculation showed only a trace of precipitin. Accordingly, the animals were given two more weekly inoculations of CB-13E in the adjuvant followed in one week by a series of four daily intraperitoneal injections of 0.5 ml. of 2 per cent CB-13E in saline solution. Serum prepared one week later showed copious precipitates with the antigen. After an interval of four days, the rabbits were again treated with four daily intraperitioneal injections of antigen in saline and were bled one week later. This latter schedule was repeated for a total of five bleedings. The quantitative precipitin content of this serum will be presented in another report.

TABLE I. STABILITY OF BUFFER SOLUTIONS TO HEAT

pH of Buffer	Composition	pH After Heating Thirty-Two Hours[1]	
		100°C	150°C
4	Acetate	—	4.0
5	Acetate	5.00	4.91
6	Acetate	5.96	5.80
7	Phosphate	6.94	7.00
8	Phosphate-borate	8.15	7.84
9	Phosphate-borate	8.85	8.92
10	Glycine-sodium hydroxide	9.84	9.60

Heated in sealed, Pyrex glass tubes.

Reaginic Serum.—Approximately 250 ml of serum was obtained from a castor bean sensitive person, E. McL. All of the tests were conducted with the same lot of serum which was stored at 5° C. Sufficient serum for two weeks testing was transferred to a small sterile vial because it was observed that repeated removals of serum from a vial over a period of four weeks caused noticeable decrease in reagin content.

Buffered Solutions of CB-1C.—One tenth molar buffer solutions were used to prepare 1 per cent solutions of CB-1C. The composition of the buffers and their stabilities (without CB-1C) when heated in sealed Pyrex tubes at 100° and 150° are shown in Table I.

Recipients for Passive Transfer Tests.—Recipients for the passive transfer tests were volunteer castor-bean nonreactors selected from among inmates of the District of Columbia Workhouse, Occoquan, Virginia. The recipients were free from antihistamine medication.

EXPERIMENTAL

Heating CB-1C Solutions.—Three ml of CB-1C in buffer solution wa: placed in a 15 x 120 mm heavy wall Pyrex tube which was sealed by flame Sealed tubes were placed in 15 ml metal centrifuge cups which were al ready at the temperature of the test. The metal cups were placed uprigh in an aluminum block 7 cm high by 14 cm in diameter which was bore to hold eight tubes and a thermometer, the block being at the test tempera ture at the start. Heating was carried out in an oven at constant temperature ± 1° C.

Preparation of Heated Solutions for Precipitin Tests.—One ml of heated CB-1C solutions was titrated to determine the amount of dilute hydrochloric acid or sodium hydroxide required to adjust the pH to between 6.5 and 7.5. The calculated amount of acid or alkali was then added to the remaining 2 ml and the volume was adjusted to 4.0 ml with buffered saline diluting fluid, giving a dilution of 1/200 (Solution A). Other desired dilutions were made with buffered saline solution, pH 7.0, for the precipitin tests.

Precipitin Test.—Fifteen hundredths ml of CB-13E rabbit antiserum in a 5 x 45 mm tube was mixed with 0.15 ml of CB-1C solution and incubated for thirty minutes at 37° C. The tubes were then placed in the refrigerator and the amount of precipitate read visually after forty-eight hours. Average of the precipitin test with unheated CB-1C diluted 1:200, $1{:}10^3$, $1{:}10^4$, $1{:}10^5$ against rabbit anti-CB-13E serum were 2+, 3+, 2+ and 1+, respectively. Control tests with unheated CB-1C in the buffer solutions against normal rabbit serum were negative.

Preparation of Solutions for Passive Transfer Tests.—Solution A, remaining after the precipitin test, was sterilized by filtration through a Pyrex bacterial filter into sterile 15 ml vials.

Reagin Neutralization Method.—Sensitized sites were located as follows: sites 1 and 2, anterior aspect of the forearm, 3½ and 1½ inches below the bend of the elbow, respectively; sites 3 and 4, upper aspect of the biceps, 2½ and 4½ inches above the bend of the elbow, respectively. Sites were used in pairs of 1 and 2, and 3 and 4 on the same arm so that 4 pairs were available, 1 pair being used for each complete test.

The reagin neutralization method is described on a day-by-day basis, four days being required for the test.

First Day, Sensitization.—Each recipient, in groups of approximately twenty-five, was sensitized by intracutaneous injection of 0.05 ml of E. McL. serum into site 1 when pair 1 and 2 was used or site 3 when pair 3 and 4 was used.

Second Day, Challenge of Site.—Site 1 or 3 was injected intracutaneously with 0.025 ml of unheated CB-1C of known concentration or with 0.025 ml of the heated CB-1C solution. The size of the wheal produced in thirty minutes was measured.

Third Day, Resensitization.—Each recipient was sensitized by intracutaneous injection of 0.05 ml of E. McL. serum in site 2 or 4 of the same arm used on the first day of the test. This site was a positive control in the test for reagin neutralization on the fourth day. This site was not sensitized on the first day because of the possibility of reagin neutralization by migration of allergen from injected site, 1 or 3.

Fourth Day, Test for Reagin Neutralization.—Each recipient was injected subcutaneously with 1.0 ml of sterile, unheated saline solution containing 1 mg of CB-1C on the outer aspect of the upper arm opposite that having the sensitized sites. This method of challenge eliminated need for a control test. Inasmuch as the sites were challenged via the body fluids, there was no trauma of sites by needle injection or irritation by solvent. The challenge dose was large enough to produce reaction with all residual reagins under the condition of the tests.

Reagin Neutralization with Unheated CB-1C.—The threshold amount of unheated CB-1C required to neutralize reagins in injected sites 1 or 3 was determined in a test using two-fold serial dilutions of CB-1C ranging from 0.13 to 512 millimicrogram/0.025 ml. A shorter range of concentrations, usually from 4 to 128 millimicrograms of unheated CB-1C/0.025 ml was used concurrently in each series of tests on the heated CB-1C solutions in order to be certain that the threshold value did not change appreciably.

Reagin Neutralization with Heated CB-1C.—The reagin neutralizing capacities of the heated CB-1C solutions were determined by injecting sites 1 or 3 on the second day of the test with 0.025 ml of sterilized Solution A which contained 125,000 millimicrograms of CB-1C.

RESULTS AND DISCUSSIONS

Results of the effect of heating solutions of CB-1C at 100, 115, and 130° C at pH values of 4, 5, 6, 7, 8, 9, and 10 from one to thirty-two hours on the precipitin reaction and on the reagin neutralizing capacity are shown in Table II. The times required to reduce the precipitating capacity from an average of 3+ at 1:1000 to a ± or doubtful value at 1:1000 dilution are shown in Column A and the times required to completely destroy precipitating capacity at 1:1000 are shown in Column B. It is evident that there is a considerable period of time during which doubtful precipitin values are obtained. For example, at 130°, pH 4, the precipitating capacity was reduced to a ± value in less than one hour, while eight hours was required to give a clear-cut negative value.

The destruction of reagin neutralizing capacity of CB-1C is shown in Column C where the time required to reduce this property to less than 0.026 per cent of its original value is recorded. A typical determination of the reagin neutralizing capacity of unheated CB-1C is shown in Table III where results with two-fold serial dilutions of CB-1C ranging from 0.13 millimicrogram to 512 millimicrograms are shown. Of nine determinations over the critical range, the neutralizing amounts of unheated CB-1C were: 32 millimicrograms, six times; 16 millimicrograms, twice; and 8 millimicrograms, once. Therefore, 32 millimicrograms of unheated CB-1C was chosen as the quantity required to neutralize reagins in 0.05

TABLE II. TIME OF HEATING REQUIRED TO DESTROY THE PRECIPITIN REACTION AND REAGIN NEUTRALIZING CAPACITY OF CB-1C
Time of Heating, in Hours

pH	Temperature °C	Precipitating Capacity		Reagin Neutralizing Capacity
		A	B	C
4	100	>32	>32	>32
	115	8	16	>32
	130	<1	8	4
5	100	>32	>32	>32
	115	8	>32	>32
	130	<1	8	4
6	100	>32	>32	>32
	115	4	8	>32
	130	4	16	4
7	100	8	16	>32
	115	2	4	32
	130	<1	2	<1
8	100	2	16	>32
	115	2	4	32
	130	<1	<1	<1
9	100	4	8	4
	115	<1	2	<1
	130	<1	<1	<1
10	100	—	2	8
	115	<1	2	<1
	130	<1	<1	—

ml of serum under the conditions of the test. Since 0.025 ml of heated CB-1C solution contained 125,000 millimicrograms, failure to neutralize reagins by a heated CB-1C solution indicated retention of less than 0.026 per cent of its original reagin neutralizing capacity.

Comparison of results in Columns A and B show that there is a considerable period of time during which ± or doubtful precipitin values are obtained before clear-cut destruction is obtained. Similarly, positive passive transfer reactions were obtained in injected sites even when the reagin neutralizing capacities were reduced to less than 0.026 per cent. This range of allergen degradation where positive direct passive transfer reactions are obtained, but where insignificant reagin neutralization occurs, may be related to that range of precipitating-antigen destruction where ± reactions are obtained. It is recognized that shocking response may be provoked by allergen which is too far degraded to neutralize reagins. It has been previously observed that in destruction of antigenic properties of acid treated cottonseed allergenic fraction CS-56 that 97 per cent of its anaphylactic sensitizing capacity was lost when only 66 per cent of its shocking capacity was destroyed.[18] The relationships of sensitizing capacity and shocking capacity of partially degraded castor bean allergen will require further study. It seems certain that a criterion indicating loss of shocking capacity would also indicate inability to sensitize, at least for components having the same specificities as found in the original castor beans. A possibility that must be borne in mind in consideration of this problem is that of the sensitization to degradation product having a new and different specificity than found in the original castor beans.

TABLE III. REAGIN NEUTRALIZING CAPACITY OF UNHEATED CB-1C

Challenge[1] Millimicrograms CB-1C/0.025 ml.	Size of Wheal in Thirty Minutes[1 2]	Test for Neutralization Reaction in Sixty Minutes[2 3]	
	Site No. 1 or 3	Site No. 1 or 3	Site No. 2 or 4[4]
0.13	±	3+	4+
.25	0	3+	3+
.50	0	4+	4+
1.0	2+	3+	2+
2.0	2+	3+	3+
4.0	2+	3+	4+
8.0	2+	2+	2+
16	2+	0	3+
32	3+	0	3+
64	3+	0	3+
128	3+	0	2+
256	3+	0	4+
512	4+	0	3+

[1] Second day test.
[2] Reactions were measured using the longest dimension of the wheal as follows: ±, questionable wheal; 1+, wheal up to 6 mm; 2+, wheal from 7 to 12 mm; 3+, wheal from 13 to 20 mm; 4+, wheals over 20 mm
[3] Fourth day test.
[4] Positive control tests.

As expected, CB-1C was more stable in acid than in alkaline solutions. Thus, at 130°, a pH of 8 or higher was required to destroy the precipitating property in less than one hour. Similarly, at 115°, a pH of 9 or higher, or at 130° a pH of 7 or higher was required to reduce the reagin neutralizing capacity of CB-1C to less than 0.026 per cent in less than one hour, respectively. In pH values of higher acidity than those, CB-1C retained demonstrable antigenic and allergenic properties in over one hour's heating at temperatures as high as 130°. Retention of these properties after such drastic heating is unique among antigens and allergens as far as published results are concerned. However, other allergens of the natural proteose classification may be equally stable, because they all retain their antigenic and allergenic properties after being heated for one hour at 100° C in water, treatment used in their isolation.[13]

SUMMARY

The effect of length of time of heating the castor bean allergen, CB-1C at 100°, 115° and 130° C in sealed tubes at pH values of 4, 5, 6, 7, 8, 9 and 10 on the destruction of precipitating capacity with rabbit castor bean allergen antiserum and reduction of *in vivo* reagin neutralizing capacity to less than 0.026 per cent of the original value with serum of a castor bean sensitive person, has been determined.

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